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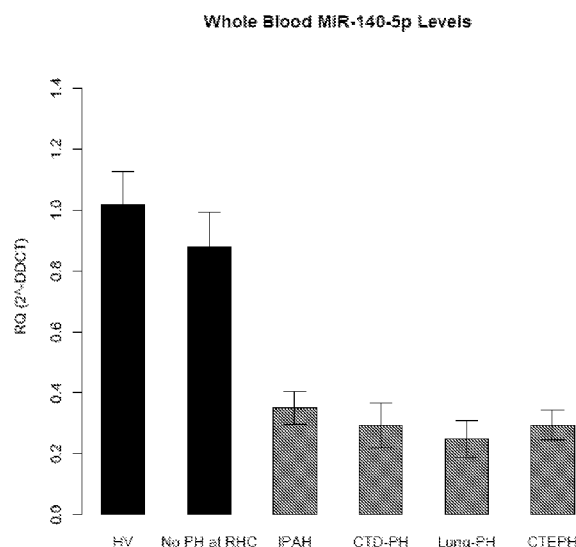
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(54) Title: BIOMARKERS FOR DISEASE STRATIFICATION

Figure 1

A



(57) Abstract: We disclose the use of miR-140-5p as a diagnostic biomarker for the detection of pulmonary hypertension (PAH) and the use of the miR in the treatment, prevention or regression of PH.



— with sequence listing part of description (Rule 5.2(a))

Biomarkers for Disease Stratification

Field of the Invention

5 The disclosure relates to a microRNA [miR] used as a diagnostic biomarker for the detection of pulmonary hypertension (PH) and the use of the miR in the treatment, prevention or regression of PH. We also disclose the use of an E3 ubiquitin protein ligase [SMURF1] either alone or in combination with said miR as a diagnostic marker of PH.

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Background to the Invention

Pulmonary Arterial Hypertension (PAH) is a devastating and debilitating disease. PAH is caused by narrowing of the arteries in the lungs resulting in abnormally high blood pressure. PAH occurs in either its idiopathic (IPAH) or hereditary (hPAH) form and is also associated with other diseases (APAH) [e.g. connective tissue disease]. Pulmonary hypertension can also result from left heart disease, lung diseases (particularly Congestive Obstructive Disease [COPD] and pulmonary fibrosis), thromboembolism as well as many other multifactorial conditions such as portal hypertension, sickle cell disease and HIV. The prognosis for patients suffering from PH is poor and varies between disease groups.

Currently there is no pharmacological cure for PH and lung transplantation is the only curative treatment. The management of PAH is less than effective and the side effect profiles of current treatments can result in further reduced quality of life. Although agents such as calcium channel blockers, diuretics, endothelin receptor antagonists, prostacyclins, soluble guanalate cyclase and phosphodiesterase inhibitors hinder the progression of the disease, the survival time after diagnosis without treatments are poor.

30 Pulmonary hypertension is extremely difficult to diagnose as the symptoms are often similar to other conditions that affect the heart or lungs. Most patients will have initially a number of tests including echocardiogram, electrocardiogram, chest X-ray, lung function tests, exercise tests, ventilation-perfusion scan and blood tests prior more accurate tests, leading to a definite diagnosis, will be offered. Accurate diagnosis of PAH, however, requires complex procedures such as right-heart catheterisation.

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The existence of an accurate marker for PAH suitable for the diagnosis of PAH would benefit patients allowing early and accurate diagnosis of the disease and a more tailored personalized treatment plan saving the health care system substantial amounts of funds and resources.

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MicroRNAs [miR] are small non-coding RNAs that control mRNA function and are involved in post transcriptional gene regulation by binding to target strands and so inhibiting translation or inducing mRNA degradation. There are a variety of processes regulated by miR such as embryonic development and alteration of miR expression has
10 been linked to tumour progression. Extracellular miRs are present in most biological fluids and are relatively stable. miR are selectively exported from cells using membrane-derived vesicles (exosomes and microparticles), lipoproteins, and other ribonucleoprotein complexes.

15 WO2012/005572 discloses the use of miR in the diagnosis of diseases or conditions associated with melanoma. WO2013/060894 discloses the use of miR for the diagnosis of atherosclerosis. The miR 140-5p has been extensively studied in zebrafish and was found to regulate the development of structures homologous to the human neurocranium. Mutations in the platelet derived growth factor receptor alpha gene
20 (*pdgfra*) in zebrafish resulted in hypoplasia of the roof of the mouth and studies confirmed that the *pdgfra* gene is regulated by the miR 140-5p. *Pdgfra*^{-/-} mice resembled a cleft face phenotype, and small nucleic polymorphisms in the miRNA gene were found to be associated with non-syndromic cleft palate patients. In our co-pending applications US14/352,120 and US14/352,126 we disclose OPG and TRAIL antibody therapy results
25 in the reversal of the symptoms of PAH. Moreover, PCT/GB2014/051205 discloses combination therapy that administers both OPG and TRAIL antibodies in the treatment of PAH. The content of US14/352,120, US14/352,126 and PCT/GB2014/051205 are incorporated by reference in their entirety.

30 We disclose decreased serum levels of miR-140-5p in patients with idiopathic and scleroderma associated PAH and the use of miR-140-5p as a therapeutic agent in the treatment of PAH, alone and in combination with further therapeutic agents effective in the treatment and reversal of PAH, and as a diagnostic biomarker of PAH.

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Statements of Invention

According to an aspect of the invention there is provided miR-140-5p, or polymorphic variant thereof, for use as a medicament.

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In a preferred embodiment of the invention miR-140-5p comprises the nucleotide sequence CAGUGGUUUUACCCUAUGGUAG [SEQ ID NO: 1], or a polymorphic sequence variant wherein SEQ ID NO: 1 is modified by addition, deletion or substitution of at least one nucleotide base and which has retained or enhanced activity associated with miR-140-5p.

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"Polymorphic variants" have high sequence identity to miR-140-5p and retain or have enhanced miR activity and which have at least 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NO: 1.

15

In a preferred embodiment of the invention miR mir-140-5p is modified.

The term "modified" as used herein describes a nucleic acid molecule in which:

20 i) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide). Alternatively or preferably said linkage may be the 5' end of one nucleotide linked to the 5' end of another nucleotide or the 3' end of one nucleotide with the 3' end of another nucleotide; and/or

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ii) a chemical group, such as cholesterol, not normally associated with nucleic acids has been covalently attached to the nucleic acid.

30 iii) Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, phosphate triesters, acetamides, peptides, and carboxymethyl esters.

The term "modified" also encompasses nucleotides with a covalently modified base and/or sugar. For example, modified nucleotides include nucleotides having sugars which are covalently attached to low molecular weight organic groups other than a

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hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleotides may also include 2' substituted sugars such as 2'-O-methyl-; 2-O-alkyl; 2-O-allyl; 2'-S-alkyl; 2'-S-allyl; 2'- fluoro-; 2'-halo or 2'-azido-ribose, carbocyclic sugar analogues a-anomeric sugars; epimeric sugars such as arabinose, xyloses or
 5 lyxoses, pyranose sugars, furanose sugars, and sedoheptulose.

Modified nucleotides are known in the art and include alkylated purines and/or pyrimidines; acylated purines and/or pyrimidines; or other heterocycles. These classes of pyrimidines and purines are known in the art and include, pseudoisocytosine; N4, N4-
 10 ethanocytosine; 8-hydroxy-N6-methyladenine; 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil; 5-fluorouracil; 5-bromouracil; 5-carboxymethylaminomethyl-2-thiouracil; 5 carboxymethylaminomethyl uracil; dihydrouracil; inosine; N6-isopentyl-adenine; 1-methyladenine; 1-methylpseudouracil; 1-methylguanine; 2,2-dimethylguanine; 2-methyladenine; 2-methylguanine; 3-
 15 methylcytosine; 5-methylcytosine; N6-methyladenine; 7-methylguanine; 5-methylaminomethyl uracil; 5-methoxy amino methyl-2-thiouracil; α -D-mannosylqueosine; 5-methoxycarbonylmethyluracil; 5-methoxyuracil; 2 methylthio-N6-isopentenyladenine; uracil-5-oxyacetic acid methyl ester; psuedouracil; 2-thiocytosine; 5-methyl-2 thiouracil, 2-thiouracil; 4-thiouracil; 5-methyluracil; N-uracil-5-oxyacetic acid methylester; uracil 5—
 20 oxyacetic acid; queosine; 2-thiocytosine; 5-propyluracil; 5-propylcytosine; 5-ethyluracil; 5-ethylcytosine; 5-butyluracil; 5-pentyluracil; 5-pentylcytosine; and 2,6,-diaminopurine; methylpsuedouracil; 1-methylguanine; 1-methylcytosine. Modified double stranded nucleic acids also can include base analogs such as C-5 propyne modified bases (see Wagner et al., Nature Biotechnology 14:840-844, 1996).

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According to a further aspect of the invention there is provided miR-140-5p for use in the treatment of pulmonary hypertension or conditions that result in pulmonary hypertension.

In a preferred embodiment of the invention miR-140-5p comprises the nucleotide
 30 sequence set forth in SEQ ID NO: 1, or a polymorphic sequence variant wherein SEQ ID NO: 1 is modified by addition, deletion or substitution of at least one nucleotide base and which has retained or enhanced activity of miR-140-5p.

In a preferred embodiment of the invention miR-140-5p treatment results in the
 35 regression of pulmonary hypertension.

In a preferred embodiment of the invention pulmonary hypertension is pulmonary arterial hypertension.

In an alternative preferred embodiment of the invention pulmonary hypertension is
5 idiopathic (IPAH) or hereditary pulmonary hypertension (hPAH).

Pulmonary hypertension can occur in various forms and is associated with a wide range of other diseases (Associated Pulmonary Arterial Hypertension: APAH) such as connective tissue disease, or is the result of left heart disease, lung diseases
10 (particularly Congestive Obstructive Disease [COPD] and pulmonary fibrosis), thromboembolism as well as may other multifactorial conditions such as portal hypertension, sickle cell disease and HIV.

According to a further aspect of the invention there is a pharmaceutical composition
15 comprising miR-140-5p, or polymorphic variant, and a pharmaceutically adjuvant and/or carrier.

In a preferred embodiment of the invention miR-140-5p comprises the nucleotide sequence set forth in SEQ ID NO: 1, or a polymorphic sequence variant wherein SEQ ID
20 NO: 1 is modified by addition, deletion or substitution of at least one nucleotide base and which has retained or enhanced activity of miR-140-5p.

In a preferred embodiment of the invention miR-140-5p is modified as herein disclosed.

25 In a preferred embodiment of the invention said pharmaceutical composition includes a carrier adapted to deliver miR-140-5p to a subject in need of treatment.

According to a further aspect of the invention there is provided a combined pharmaceutical composition comprising miR-140-5p and at least one additional
30 therapeutic agent used in the treatment of PAH.

In a preferred embodiment of the invention miR-140-5p comprises a nucleotide sequence set forth in SEQ ID NO: 1, or a polymorphic sequence variant wherein SEQ ID NO: 1 is modified by addition, deletion or substitution of at least one nucleotide base and
35 which has retained or enhanced activity associated with miR-140-5p.

In a preferred embodiment of the invention said additional therapeutic agent is selected from the group consisting of: calcium channel blockers, diuretics, endothelin receptor antagonists, prostacyclins, soluble guanalate cyclase and phosphodiesterase inhibitors.

- 5 In alternative preferred embodiment of the invention said additional therapeutic agent is an antagonistic antibody, or active binding fragment thereof, that binds and inhibits the activity of Tumour Necrosis Factor Apoptosis-Inducing Ligand [TRAIL].

- 10 In a preferred embodiment of the invention said antibody competes with an antibody that binds to the amino acid sequence as represented in SEQ ID NO: 2, 3 or 4.

In a preferred embodiment of the invention said antibody or binding fragment binds the extracellular domain of TRAIL.

- 15 In a preferred embodiment of the invention said antibody binds an epitope located between amino acid residues 91–281 of the amino acid sequence presented in SEQ ID NO: 2.

- 20 In a further alternative preferred embodiment of the invention said additional therapeutic agent is an antagonistic antibody, or active binding fragment thereof, that binds and inhibits the activity of osteoprotegerin [OPG].

- 25 In a preferred embodiment of the invention said antibody competes with an antibody that binds to the amino acid sequence as represented in SEQ ID NO: 5.

In a preferred embodiment of the invention said antibody is a polyclonal antibody.

- 30 In an alternative preferred embodiment of the invention said antibody is a monoclonal antibody.

- 35 Antibodies, also known as immunoglobulins, are protein molecules which have specificity for foreign molecules (antigens). Immunoglobulins (Ig) are a class of structurally related proteins consisting of two pairs of polypeptide chains, one pair of light (L) (low molecular weight) chain (κ or λ), and one pair of heavy (H) chains (γ , α , μ , δ and ϵ), all four linked together by disulphide bonds. Both H and L chains have regions that contribute to the binding of antigen and that are highly variable from one Ig molecule to another. In

addition, H and L chains contain regions that are non-variable or constant. The L chains consist of two domains. The carboxy-terminal domain is essentially identical among L chains of a given type and is referred to as the "constant" (C) region. The amino terminal domain varies from L chain to L chain and contributes to the binding site of the antibody. Because of its variability, it is referred to as the "variable" (V) region. The H chains of Ig molecules are of several classes, α , μ , σ , α , and γ (of which there are several sub-classes). An assembled Ig molecule consisting of one or more units of two identical H and L chains, derives its name from the H chain that it possesses. Thus, there are five Ig isotypes: IgA, IgM, IgD, IgE and IgG (with four sub-classes based on the differences in the H chains, i.e., IgG1, IgG2, IgG3 and IgG4). Further detail regarding antibody structure and their various functions can be found in, Using Antibodies: A laboratory manual, Cold Spring Harbour Laboratory Press.

In a preferred embodiment of the invention said fragment is a single chain antibody fragment.

Various fragments of antibodies are known in the art, e.g. Fab, Fab₂, F(ab')₂, Fv, Fc, Fd,, etc. A Fab fragment is a multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region, covalently coupled together and capable of specifically binding to an antigen. Fab fragments are generated via proteolytic cleavage (with, for example, papain) of an intact immunoglobulin molecule. A Fab₂ fragment comprises two joined Fab fragments. When these two fragments are joined by the immunoglobulin hinge region, a F(ab')₂ fragment results. An Fv fragment is multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region covalently coupled together and capable of specifically binding to an antigen. A fragment could also be a single chain polypeptide containing only one light chain variable region, or a fragment thereof that contains the three CDRs of the light chain variable region, without an associated heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multi specific antibodies formed from antibody fragments, this has for example been described in US patent 6,248,516. Fv fragments or single region (domain) fragments are typically generated by expression in host cell lines of the relevant identified regions. These and other immunoglobulin or antibody fragments are within the scope of the invention and are described in standard immunology textbooks such as Paul, *Fundamental Immunology* or Janeway's Immunobiology, Murphy, K., Travers, P. & Walport P. Molecular biology now allows

direct synthesis (via expression in cells or chemically) of these fragments, as well as synthesis of combinations thereof. A fragment of an antibody or immunoglobulin can also have bispecific function as described above.

- 5 In a preferred embodiment of the invention said antibody is a chimeric antibody.

In an alternative preferred embodiment of the invention said antibody is a humanized or human antibody.

- 10 Chimeric antibodies are recombinant antibodies in which all of the V-regions of a mouse or rat antibody are combined with human antibody C-regions. Humanised antibodies are recombinant hybrid antibodies which fuse the complementarity determining regions from a rodent antibody V-region with the framework regions from the human antibody V-regions. The C-regions from the human antibody are also used. The complementarity
15 determining regions (CDRs) are the regions within the N-terminal domain of both the heavy and light chain of the antibody to where the majority of the variation of the V-region is restricted. These regions form loops at the surface of the antibody molecule. These loops provide the binding surface between the antibody and antigen.
- 20 Antibodies from non-human animals provoke an immune response to the foreign antibody and its removal from the circulation. Both chimeric and humanised antibodies have reduced antigenicity when injected to a human subject because there is a reduced amount of rodent (i.e. foreign) antibody within the recombinant hybrid antibody, while the human antibody regions do not elicit an immune response. This results in a weaker
25 immune response and a decrease in the clearance of the antibody. This is clearly desirable when using therapeutic antibodies in the treatment of human diseases. Humanised antibodies are designed to have less "foreign" antibody regions and are therefore thought to be less immunogenic than chimeric antibodies.
- 30 When administered the compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers and supplementary therapeutic agents.
- 35 The compositions of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for

example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, transdermal or trans-epithelial.

The compositions of the invention are administered in effective amounts. An “effective amount” is that amount of a composition that alone, or together with further doses, produces the desired response. In the case of treating a particular disease, such as PAH, the desired response is inhibiting or reversing the progression of the disease. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently and ideally reversing disease phenotype. This can be monitored by routine methods.

Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, the individual patient parameters including age, physical condition, size and weight, the duration of the treatment, the nature of concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is generally preferred that a maximum dose of the individual components or combinations thereof be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

The pharmaceutical compositions used in the foregoing methods preferably are sterile and contain an effective amount of miR according to the invention for producing the desired response in a unit of weight or volume suitable for administration to a patient.

The doses of the miR according to the invention administered to a subject can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject. Other factors include the desired period of treatment. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits.

In general, doses of miR of between 1nM - 1μM generally will be formulated and administered according to standard procedures. Preferably doses can range from 1nM-

- 500nM, 5nM-200nM, and 10nM-100nM. Other protocols for the administration of compositions will be known to one of ordinary skill in the art, in which the dose amount, schedule of injections, sites of injections, mode of administration and the like vary from the foregoing. The administration of compositions to mammals other than humans, (e.g.
- 5 for testing purposes or veterinary therapeutic purposes), is carried out under substantially the same conditions as described above. A subject, as used herein, is a mammal, preferably a human, and including a non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent.
- 10 When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. Such preparations may routinely contain salts, buffering agents, preservatives, compatible
- 15 carriers, and optionally other therapeutic agents' used in the treatment of PAH. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but
- 20 are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.
- 25 Compositions may be combined, if desired, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" in this context denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to
- 30 facilitate the application, (e.g. liposome or immuno-liposome). The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.
- 35 The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

5 The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the
10 product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous
15 liquids such as syrup, elixir or an emulsion or as a gel. Compositions may be administered as aerosols and inhaled.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous or non-aqueous preparation of agent, which is preferably isotonic with the
20 blood of the recipient. This preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1, 3-butane diol. Among the acceptable solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally
25 employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in
30 Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

According to a further aspect of the invention the pharmaceutical composition is adapted to be delivered as an aerosol.

35 According to a further aspect of the invention there is provided an inhaler comprising a pharmaceutical composition according to the invention.

According to a further aspect of the invention there is provided a diagnostic method for determining if a subject has or is predisposed to pulmonary hypertension comprising:

- i) providing an isolated biological sample to be tested;
- 5 ii) forming a preparation comprising said nucleic acid to be detected in said sample, an oligonucleotide probe or probes adapted to anneal to a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 1; a thermostable DNA polymerase, deoxynucleotide triphosphates and co-factors;
- 10 iii) providing polymerase chain reaction conditions sufficient to amplify all or part of said nucleic acid molecule;
- iv) analysing the amplified products of said polymerase chain reaction for the presence of a nucleic acid molecule comprising a nucleotide sequence derived from SEQ ID NO: 1; and optionally
- v) comparing the amplified product with a normal matched control.

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The levels of miR-140-5p are decreased by at least 2 fold when compared to a normal matched control. More particularly the levels are decreased by 3, 4, 5, 6, 7, 8, 9 or at least 10-fold compared to a normal control level of miR-140-5p.

20 According to an alternative aspect of the invention there is provided a diagnostic method for determining if a subject has or is predisposed to pulmonary hypertension comprising:

- i) providing an isolated biological sample to be tested;
- 25 ii) forming a preparation comprising said nucleic acid to be detected in said sample, an oligonucleotide probe or probes adapted to anneal to a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 6; a thermostable DNA polymerase, deoxynucleotide triphosphates and co-factors;
- iii) providing polymerase chain reaction conditions sufficient to amplify all or part of said nucleic acid molecule;
- iv) analysing the amplified products of said polymerase chain reaction for the
30 presence of a nucleic acid molecule comprising a nucleotide sequence derived from SEQ ID NO: 6; and optionally
- v) comparing the amplified product with a normal matched control.

35 In a preferred method of the invention said method is a real time PCR method for the detection and quantification of a nucleic acid encoding all or part of the nucleotide sequence set forth in SEQ ID NO: 1 or 6.

According to an alternative aspect of the invention there is provided a diagnostic method for determining if a subject has or is predisposed to pulmonary hypertension comprising the steps of:

- 5 i) providing an isolated biological sample to be tested;
- ii) forming a preparation comprising said sample and an antibody, or antibodies, that specifically bind a polypeptide in said sample as represented by the amino acid sequences presented in SEQ ID NO: 7 to form an antibody/polypeptide complex;
- 10 iii) detecting the complex; and
- iv) comparing the expression of said polypeptide with a normal matched control.

15 The level of SMURF1 mRNA [SEQ ID NO: 6] or protein [SEQ ID NO: 7] is increased by at least 2 fold when compared to a normal matched control. More particularly the levels are increased by 3, 4, 5, 6, 7, 8, 9 or at least 10-fold compared to a normal control level of SMURF mRNA or protein.

20 In a preferred method of the invention said method further includes the detection of the expression of one or more genes associated with the BMP and/or PDGF pathway.

 Preferably said gene is selected from the group consisting of: SMURF1, WWP2, RUNX2, BMP, PDGFRA, OPG, SP1, TRAIL, ID1, TGRBR1 or VEGFA.

25 In a preferred embodiment of the invention said isolated biological sample is selected from the group consisting of: blood, blood plasma or serum, lymph fluid, saliva, sputum, lavage, bronchoalveolar lavage, or human tissue biopsy.

30 In a preferred method of the invention said biological sample is a blood sample.

 In a preferred method of the invention said biological sample is a peripheral blood sample.

35 In an alternative preferred method of the invention said sample is a tissue biopsy. Preferably said biopsy is a lung tissue biopsy.

In a preferred method of the invention said method is combined with an alternative diagnostic method for the detection of pulmonary hypertension in a subject.

5 In an alternative preferred method of the invention said method is selected from the group echocardiogram, electrocardiogram, chest X-ray, lung function tests, exercise tests, ventilation-perfusion scan, blood tests or right heart catheterisation.

According to a further aspect of the invention there is provided a treatment method for PAH comprising:

- 10 i) conducting a diagnostic assay according to the invention on a subject to be tested for PAH;
- ii) determining whether the subject needs therapeutic intervention; and if requiring therapeutic intervention,
- iii) administering a medicament or pharmaceutical composition according to
- 15 the invention to the subject.

In a preferred method of the invention said medicament comprises miR 140.5p.

20 In an alternative preferred method of the invention said subject is administered a second medicament or pharmaceutical composition comprising one or more agents used in the treatment of PAH.

25 In a preferred method of the invention said agent[s] are selected from the group calcium channel blockers, diuretics, endothelin receptor antagonists, prostacyclins, soluble guanalate cyclase and phosphodiesterase inhibitors.

30 In alternative preferred method of the invention said agent[s] is an antagonistic antibody, or active binding fragment thereof, that binds and inhibits the activity of Tumour Necrosis Factor Apoptosis-Inducing Ligand [TRAIL] or osteoprotegerin as herein disclosed.

In a preferred method of the invention said medicaments or compositions are administered simultaneously or sequentially.

35 In a preferred method of the invention pulmonary hypertension is pulmonary arterial hypertension.

In an alternative preferred method of the invention pulmonary hypertension is idiopathic (IPAH) or hereditary pulmonary hypertension (hPAH).

Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of the words, for example “comprising” and “comprises”, means “including but not limited to”, and is not intended to (and does not) exclude other moieties, additives, components, integers or steps. “Consisting essentially” means having the essential integers but including integers which do not materially affect the function of the essential integers

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

An embodiment of the invention will be described by example only and with reference to the following figures:

Figure 1 A) Levels of miR-140-5p are reduced in patients with PH (idiopathic PH, PAH associated with connective tissue disease (CTD-PH) and lung disease (Lung-PH), and Chronic Thromboembolic Pulmonary Hypertension (CTEPH)) compared to healthy volunteers (HV) and patients with connective tissue disease without PAH. B) Levels of miR-140-5p reduce with increasing mean pulmonary artery pressure (mPAP) (B), pulmonary vascular resistance (PVR) (C) and the cardiac stress marker N-terminal Pro=brain natriuretic peptide (NT-pro-BNP) (D). E) Patients with low levels of miR-140-5p at diagnosis suffer a reduced survival at 2 years. F) Transfection of the pre-miR-140-5p mimic induces a decrease in the E3 ubiquitin ligase SMURF1. H) Whole blood mRNA levels of ID1 mRNA are higher (G) and its downstream target SMURF1 are lower (H) at diagnosis in patients with IPAH. RVSP (Right Ventricular Systolic Pressure) (I) and RVH (Right ventricular hypertrophy) (J) in the monocrotaline rat model of PAH (with saline injected control). K) Levels of miR-140-5p are reduced in the monocrotaline (K; lung)

and sugen hypoxia (L; whole blood) rat models of PAH during the onset and progression of PAH.

Figure 2 A) Administration of nebulised pre-miR-140-5p prevents disease development in the monocrotaline rat: A) Pulmonary artery acceleration time (PAAT), B) RVSP C) CO. D) Whole lung miR140-5p is increased with administration of pre-miR-140-5p (Pre) compared to the Anti-miR-140-5p (Anti) and scrambled (SCR) molecules. E) Whole lung mRNA levels of SMURF1. Administration of nebulised pre-miR-140-5p reverses established disease in the monocrotaline rat: F) PAAT, G) RVSP H) CO. I) Levels of miR140-5p are increased with administration of pre-miR-140-5p. Administration of nebulised pre-miR-140-5p reverses established disease in the Sugan hypoxic rat: K) RVSP and K) CO. L) Levels of miR140-5p are increased with administration of pre-miR-140-5p. M) Whole lung mRNA levels of SMURF1 are reduced with administration of pre-miR-140-5p.

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Figure 3 Suppression of miR-140-5p with an anti-miR-140-5p oligo induces further proliferation of human PSMCs in response to serum (A), and spontaneous proliferation in serum free conditions, which is then inhibited with SMURF1 siRNA (B); C) Western blot measuring protein expression of PDGFA normalised to the endogenous control gene GAPDH following the transfection of human PSMC with either the pre- or anti-miR-140-5p oligo. Cell transfected with the pre-miR-140-5p display a reduced expression of PDGFRA, whereas cells transfected with the anti-miR-140-5p demonstrate increased expression compared to a scrambles molecule (SCR).

Figure 4 Treatment of established PAH (8 week Paigen diet) with an anti-TRAIL antibody induces reverse remodelling of disease: ApoE^{-/-} mice with either anti-TRAIL antibody of IgG for 4 weeks. Mice that received the anti-TRAIL antibody displayed a significant reduction in pulmonary vascular remodelling as shown by the reduction in media/CSA of the small pulmonary arteries/arterioles (Figure 4A). This was associated with decrease in proliferating (PCNA) cells (Figure 4B) and an increase in apoptotic (TUNEL) cells within remodelled pulmonary arteries (Figure 4C).

Figure 5 Treatment of established PAH (8 week Paigen Diet) with an anti-OPG antibody induces reverse remodelling of disease: ApoE^{-/-} mice with anti-OPG antibody of IgG for 4 weeks. Mice that received the anti-OPG antibody displayed a significant reduction in pulmonary vascular remodelling as shown by the reduction in media/CSA of the small

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pulmonary arteries/arterioles (Figure 5A). This was associated with decrease in proliferating (PCNA) cells (Figure 5B) and an increase in apoptotic (TUNEL) cells within remodelled pulmonary arteries (Figure 5C). The Anti-OPG treatment demonstrates a more pronounced pro-apoptotic response than anti-TRAIL, which was associated with a greater anti-proliferative response.

Materials and Methods

Animals

10 Monocrotaline Rats

Outbred male, albino Sprague Dawley rats (Charles River or Harlan, U.K) (starting weight approx. 200g) were used for experiments. A single subcutaneous injection of monocrotaline (MCT) (see below) into the left thigh was used to induce pulmonary arterial hypertension. We used a well established dose of 60mg/kg that leads to the development of severe PAH and which is fatal within 5-6 weeks. 200mg of Monocrotaline (MCT) (#C2041-500, Sigma Aldrich, UK) was first fully dissolved in 0.6ml of 1M Hydrochloric Acid and vortexed for 40min. Sterile water was added to make the volume to 5ml and the pH adjusted to 7.0 with sterile NaOH. A final solution to 10ml was made with sterile water.

20

Sugen Hypoxia Rats

Experiments used outbred male, albino Wistar rats (Harlan Laboratories, UK) (starting weight 200-250 g). Vehicle was prepared by adding 0.5 g CMC (0.5% (wt/vol) carboxyl methylcellulose sodium, Sigma Aldrich, UK), 400 µl Tween 80 (0.4% (vol/vol) polysorbate 80, Sigma Aldrich, UK) and 900µl benzyl alcohol to 98.7 ml of 0.9% (wt/vol) Sodium chloride (Sigma Aldrich, UK) and stirring on a warming plate until clear. 5 mg/ml Sugén was prepared by adding 2 ml of vehicle to 10 mg of Sugén 5416 (Tocris, UK) and sonicating in a water bath for 15 min. 0.6 ml of Sugén 5416 (20 mg/kg) was administered to each rat (200-250 g) by sub cutaneous injection. Rats were housed in hypoxic chambers at 10% O₂ and 5% CO₂ for 3 weeks and in normoxia for 5 weeks for experimentation or 11 weeks of normoxia for time course experiments.

Animal care and investigation conformed to the University's ethical policy statement and the UK Home office guidance in the operation of Animal Scientific Procedures Act 1986 on H.O. project license PPL 40/2952 (Held by Lawrie).

Disease Phenotyping

Each rodent underwent cardiac catheterization and was then sacrificed whilst still under anaesthesia. The abdominal aorta was cut and lungs were perfused with PBS via a
5 needle in the right ventricle until the lungs became visibly white. The heart and lungs were removed en-bloc. The right lung was quickly separated before immediately being snap frozen in liquid nitrogen for subsequent biochemical analyses. The left lung was perfusion fixed, via the trachea with 10% (v/v) formalin at an inflation pressure of 20cm H₂O and then placed with the heart in 10% formalin overnight at 4°C. The left lung was
10 subsequently used for histological and immunohistochemical analyses.

Rat Study Protocol

Rodent cardiac catheterisation

Left and right ventricular catheterisation was performed using a closed chest method via
15 the right internal carotid artery and right external jugular vein under isoflurane induced anaesthesia. Data was collected using a Millar ultra-miniature pressure-volume SPR-838 (Millar Instruments Inc., Texas, USA) coupled to a Millar MPVS 300 and a PowerLab 8/30 data acquisition system (AD Instruments, Oxfordshire, UK) and recorded using Chart v7 software (AD Instruments). Pressure volume analysis was performed using
20 PVAN v2.3 (Millar Instruments Inc).

Harvesting and processing of tissue

Lung tissue protocol

Following sacrifice an incision in the upper abdominal wall was made to expose the liver. Whilst applying upward traction on the xiphoid process of the sternum, the diaphragm
25 was carefully cut with fine scissors. The sternum and chest wall were resected away. The abdominal aorta was identified and cut (to exsanguinate). Using a 25G orange needle and syringe the right ventricle was identified and flushed with PBS until the lungs became pale. The trachea was identified and freed between the medial clavicular borders. Whilst applying firm upward traction on the trachea, the heart and lungs were
30 removed en-bloc from the posterior wall of thoracic cavity. Care was taken to avoid inadvertent lung puncture. The right lung was secured tightly at the hilum using 5-0 silk sutures and separated away before being snap frozen in liquid nitrogen for subsequent isolation and determination of whole lung protein and RNA expression. Polyethylene tubing was inserted into the trachea and secured tightly with a suture. The left lung was
35 gently inflated manually with a syringe containing 10% phosphate buffered formalin

(0.4% w/v $\text{NaH}_2\text{PO}_4 \cdot 2(\text{H}_2\text{O})$, 0.65% w/v $\text{Na}_2\text{HPO}_4 \cdot 2(\text{H}_2\text{O})$ and 4% v/v formaldehyde in water) and then both heart and left lung were fixed in formalin for 24 hours before transfer into PBS. From the rat prevention study onwards lungs were inflated using 20cm H_2O clamp set up to standardise inflation. The left lung was separated from the heart for subsequent histology.

Heart weights and right ventricle hypertrophy (RVH) - protocol

RVH was defined as the weight of the RV divided by the weight of the left ventricle/septum (RV/LV+S) as first described by Fulton et al. Using a small pair of fine scissors surrounding fat, tissue and great vessels were removed from around the heart. The atria were excised, cleared of any thrombus and weighed. The right ventricle was separated from the left ventricle and septum by the use of anatomical landmarks.

Starting from the right ventricular outflow tract (RVOT) the septal margin of the RV was dissected away to ensure no ridges of tissue were left. An incision was also made from the RVOT adjacent to and encircling the aortic root towards the medial tricuspid valve annulus to separate the base of the RV. From the lateral tricuspid annulus the RV free wall was cut away ensuring again no ridges of RV tissue remained. The incision continued towards the apex and back up-towards the RVOT. Finally the left ventricle was cut and any clot removed from it before all chambers were padded dry and weighed.

IMMUNOHISTOCHEMISTRY

Paraffin embedded 5µm lung sections underwent immunohistochemical staining α-SMA for vascular smooth muscle cells, vWF to localise endothelial cells and PCNA for proliferating cells. Immunostaining for OPG was performed to identify any expression within pulmonary vascular lesions. Levels of apoptosis were determined with a colorimetric assay to detect DNA fragmentation (FRAGEL®, Calbiochem, UK) as specified by the manufacturer's instructions. A positive control was generated with DNase treatment of a control slide.

Protocol

Following de-waxing and rehydration of slides, endogenous tissue peroxidases were blocked by incubating slides in 3% (v/v) hydrogen peroxide for 10mins before being rinsed in tap water. Antigen retrieval (Slide permeabilisation) was done by incubating slides in either:

a) citrate buffer, pH 6.0 preheated to 95°C for 20min. before cooling for 20min at RT. Tissue was then permeabilised by incubation in 0.5% (v/v) tritonX100 for 10mins at RT (IHC for OPG)

5

b) 0.1% (w/v) Trypsin/TBS, pH7.8, preheated to 37°C for 10minutes before stopping reaction by immersing in water (IHC for vWF)

c) For SMA staining an antigen retrieval step was not performed

d) EDTA buffer, pH 8.0 preheated to 95°C for 20min before cooling for 20min at RT.

10 Tissue was then permeabilised by incubation in 0.5% (v/v) tritonX100 for 10mins at RT (IHC for SMURF1)

Slides were then blocked (to prevent non-specific binding of secondary antibody) in 1% (w/v) skimmed milk/PBS for 30mins at RT. Milk was tipped off and excess blotted away.

15 The relevant primary antibody diluted in PBS was added and incubated as follows:

a) Monoclonal mouse anti-human aSMA 1:150, (#m081, Dako) for 1 hour at RT

b) Polyclonal rabbit anti-human vWF 1:300 (#A0082, Dako) for 1hour at RT.

c) Polyclonal rabbit anti-human OPG 1:100 (#ab73400, Abcam) overnight at 4C

20 d) Polyclonal mouse anti-human SMURF1 1:100 (#ab57573, abcam overnight at 4°C.

Time Course

Monocrotaline Time Course

25

In time course experiments rats (200-260g, n=7/group/time-point) underwent haemodynamic study and sacrifice either 2,7,14,21 or 28 days after injection with MCT (60mg/kg) or saline control.

Interventions

30 Monocrotaline Prevention Study

To investigate whether loss of miR-140-5p was required for, or if an increase of miR-140-5p was protective from, the development of disease in the MCT model SD rats (weight 280-300 g, n=8 per group) were treated with two weekly doses of 20 µM SCR, pre-140-5p or anti-miR-140-5p by intra tracheal nebulisation starting at day 7 and day 14 post injection with MCT. Disease phenotyping and tissue harvest was undertaken 21 days post MCT injection.

35

Monocrotaline Therapeutic Study

To investigate the effect on established disease miR-140-5p was delivered by intra-tracheal nebulisation to SD rats at 21 days post MCT. Monocrotaline was injected into the right thigh of SD rats as described at day 0 (weight 280-300g, n=8 per group).

- 5 Confirmation of disease development was made by echocardiogram at day 21. Two, weekly doses of 20 μ M SCR, pre-140-5p or anti-miR-140-5p were administered by intra tracheal nebulisation at day 21 and day 28 and disease phenotyping and tissue harvest was undertaken at 35 days post MCT injection.

10 Sugén Therapeutic Study

To investigate the effect on established disease miR-140-5p was delivered by intra-tracheal nebulisation to SD rats 56 days post sugen injection. Sugén was injected into the right thigh of SD rats at day 0 (weight 200-250 g, n=8 per group) and animals housed in hypoxic chambers for 3 weeks as described above. Confirmation of disease
15 development was made by echocardiogram at day 56. Two, weekly doses of 20 μ M SCR, pre-140-5p or anti-miR-140-5p were administered by intra tracheal nebulisation at day 56 and day 63 and disease phenotyping and tissue harvest was undertaken at day 70 post sugen injection.

20 Nebulisation Interventions

Preparation of In Vivo Transfection Mixture

- 3 mg/L (200 M) stock was prepared by re-suspending 250 nmol of HPLC miR/SCR (Invitrogen, UK) in 1.25 mL of DNase RNase free water (Ambion, UK). Using the stock miR/SCR an in vivo transfection mixture (10 doses of 20 nM in 100uL) was prepared by
25 mixing two transfection reagents tubes (Tube 1: Mix 100 μ L of 200 μ M miR/SCR stock with 100 μ L of Complexation Buffer (Invivofectamine, Invitrogen, UK); Tube 2: 200 μ L of Invivofectamine (Invitrogen, UK) at room temperature) then vortexing for 2-3 seconds prior to incubation for 30 min at 50°C. During transfection mixture incubation a Float-A-Lyzer dialysis filter (Spectrum Medical Laboratories, UK) was prepared by filling the filter
30 with and submerging in 10% (v/v) isopropanol for 10 min. Alcohol was removed by aspiration and the Float-A-Lyzer flushed, filled and soaked in deionized water for 15-20 min. Deionized water was then aspirated and the Float-A-Lyzer loaded with transfection mixture by pipetting to the bottom of the membrane slowly withdrawing pipet as dispensing. The Float-A-Lyzer (with ring float) was placed vertically in 1L of PBS and
35 incubated for 2 hours with gentle agitation. The sample was collected by aspirating while

moving pipette toward bottom of membrane and marked up to 2 mL with PBS in sterile tube.

Nebulised Intra-Tracheal Delivery of In Vivo Transfection Mixture

- 5 Rodents were anaesthetised with isoflurane (5%) via oxygen (2 L/min) in an induction chamber (Harvard Apparatus, UK) and placed supine on a heated platform at 45° with the head orientated towards the operator. Animals were covered to minimise heat loss and maintenance isoflurane (0.5-1.5%) delivered with oxygen (1 L/min) via a nose cone. Continuous heart rate monitoring was used to monitor depth of anaesthesia. The nose
10 cone was removed to allow delivery of transfection reagent, the vocal cords were visualised with a small animal laryngoscope (Model LS-2, Penn-Century, USA) and the animal intubated with a MicroSprayer Aerosolizer (Model LA-1B, Penn-Century, USA). 20 µM of miR/SCR was delivered via a 250 µL high-pressure syringe (FMJ-250, Penn-Century, USA).

15

Interventions

- Where stated polyclonal goat anti-mouse TRAIL (Anti-TRAIL), polyclonal goat anti-mouse OPG (Anti-OPG) or control goat IgG isotype antibodies (R&D systems, UK) where delivered to rodents through subcutaneously implanted osmotic pumps (Durect
20 Corp., CA, USA). Interventions were delivered via an Alzet® 1004 micro pump (100µl reservoir, 0.1µl/hour for 4 weeks) in mice and via an Alzet® 2002 mini-pump (200µl reservoir, 0.5µl/hr, 85ng/hr for 2 weeks) in rats.

Pump Implantation protocol

- 25 Each pump was filled with the appropriate intervention under sterile conditions in a class II laminar flow hood and placed in sterile 0.9% saline at 37°C 24 hours prior to implantation. Under isoflurane gas anaesthesia (2-3%, IsoFlo® 100% w/w inhalation vapour liquid, Abbot laboratories Ltd, Kent, UK) through 100% oxygen (flow rate 1.5L/min) overlying fur was clipped, the skin cleaned and sterilised prior to making a 1-
30 1.5cm cutaneous incision over the left posterolateral thoracic wall, inferior to the lower costal margin. Under sterile surgical conditions, pre-filled pumps were implanted into a subcutaneous pocket created with blunt dissection. The wound was subsequently cleaned and closed using interrupted 2-0 Vicryl absorbable sutures (B-Braun, Sheffield, UK). Implantation for mice was identical except pumps were primed for 48 hours,
35 implanted posterior to the cervical spine (scruff line) and wounds closed with interrupted Silk sutures (Silkam®, B-Braun Sheffield, UK).

Experimental Protocol

Mice

5 ApoE^{-/-}, TRAIL^{-/-} and ApoE^{-/-}/TRAIL^{-/-} knockout mice (10-16 weeks of age, n=4-7/group) where fed either chow or Paigen for 8 weeks before disease phenotyping (see below). In separate experiments ApoE^{-/-}/TRAIL^{-/-} mice (8-12 weeks of age, n=4-6/group) were treated with either rmTRAIL (10ng/hr) or placebo (PBS) by osmotic pumps for 4 weeks that coincided with the onset of feeding Paigen diet.

10

To determine the efficacy of inhibiting TRAIL in mice with established disease, ApoE^{-/-} mice (8-10 weeks of age, n=6-7/group) were fed a Paigen diet for 8 weeks and then received an anti-TRAIL antibody (20ng/hr) or isotype control with phenotyping performed at week 12.

15

ApoE^{-/-} knockout mice (10-16 weeks of age, n=4-7/group) where fed either chow or Paigen for 8 weeks before disease phenotyping (see below). To determine the efficacy of inhibiting OPG in mice with established disease, ApoE^{-/-} mice (8-10 weeks of age, n=6-7/group) were fed a Paigen diet for 8 weeks and then received an anti-OPG
20 antibody (20ng/hr) or isotype control with phenotyping performed at week 12.

Rats

To investigate if TRAIL or OPG was required for the development of disease (Prevention study) rats (200-240g, n=4/group) were treated with an anti-TRAIL antibody (84ng/hr) or
25 isotype control delivered for 2 weeks by osmotic pumps, commencing at baseline with MCT injection. Disease phenotyping was undertaken one week later, i.e.: 21 days after MCT injection.

To determine the efficacy of inhibiting TRAIL or OPG in established disease (Survival study), rats (200-240g, n=6/group) with MCT induced PAH (day 21 after MCT) received
30 an anti-TRAIL antibody (84ng/hr) or isotype control for 2 weeks. We compared survival in the two groups and rats were sacrificed at day 35 post MCT (1-14 days following intervention) or sooner if they displayed morbidity and evidence of right heart failure. The latter were defined by outward illness (breathlessness, lethargy, ruffled fur) AND
35 significant weight loss (defined as >5% weight loss in 24 hours or a total of 10% over 48

hours). Where possible, echocardiographic and haemodynamic studies were performed immediately prior to sacrifice.

Isolation and purification of RNA from lung tissue - protocol

- 5 Lung segments frozen in liquid nitrogen were ground using a pestle and mortar containing liquid nitrogen to a fine powder and weighed. Precautions were taken to minimise contamination by RNAase. Total protein and RNA were isolated using a commercial RNA/Protein purification Kit (#23000, Norgen Biotek, Ontario, Canada) according to the protocol supplied by the manufacturer. The purification kit employed a
10 spin column chromatography technique and allowed elution of proteins and RNA from the same sample within 30 minutes.

- Briefly lysis solution was added to the lung tissue and then ethanol added. This was loaded on to a spin-column. After centrifugation at 14000rpm, all nucleic acids within the
15 solution were bound by a resin whilst the proteins were removed in the flow through. The bound RNA was washed, spun again and then purified RNA was eluted. The concentration of RNA in the elution was determined using a spectrophotometer (NanaDrop®, Thermo Scientific) and frozen at -80C.

20 Human Pulmonary artery Smooth Muscle cell Studies

Transfection of pre- and anti-miR-140-5p

- Human pulmonary artery smooth muscle cells (PA-SMC) were purchased from Lonza and cultured as per supplier instructions. Cell between passage 5-7 were transfected with either scrambled, pre-miR-140-5p or anti-miR-140-5p oligos (Ambion/Life
25 Technologies) using SuperFect (Qiagen) in the presence or absence of full growth media.

Cell Phenotype Assays

- Assessment of proliferation was determined by performing cell counts on a Coulter
30 Counter (Coulter, USA) and expressed as Cell number.

SMURF 1 proteins detection - protocol

- Analysis of SMURF1 protein levels, a putative target of miR-140-5p were assessed by western immunoblotting. Proteins were isolated using RIPA buffer and separated by
35 SDS-Polyacrylamide gel electrophoresis using a commercial electrophoresis kit (NuPAGE® Kit, Invitrogen). All buffers and reagents were part of the NuPAGE range

unless otherwise stated. A volume containing 35 µg of protein purified from control, pre- and anti miR-140p transfected human pulmonary artery smooth muscle cells, sample buffer and a reducing agent made to a final volume of 30 µl (in deionised water) was heated to 70°C for 10 min. Samples and a pre-stained marker ladder were then loaded
5 onto 10 well pre-cast SDS polyacrylamide gels (NuPAGE® 4-12% Bis-Tris Mini gels, Invitrogen). In addition a sample of mixed experimental lung tissue was also loaded onto every gel as an additional control to allow for subsequent quantitative analysis.

Immediately prior to placing the loaded gels into an electrophoresis cell (XCell
10 SureLock® Mini cell, Invitrogen) that already contained SDS running buffer, 500 µl of antioxidant was added. The Gel was run at 200V for 35 min.

Gels were transferred onto a nitrocellulose membrane (membrane and blotting pads had been pre-soaked in the transfer buffer and air bubbles removed) in transfer buffer
15 (containing antioxidant and 10% methanol v/v) and ran at 30V for 60min. Ponceau S staining was used to confirm adequate transfer.

The membranes were then blocked for 1h in 10ml of PBS with 5% milk (w/v) and 0.1% Tween-20 (v/v) on a shaking platform. Blots were rinsed in PBS/0.1% tween-20 three
20 times before adding the relevant primary antibody in 5% milk/PBS/0.1% Tween-20 on a shaking platform overnight at 4°C. (Rabbit anti-human SMURF1 1:1000, #2174, Cell Signalling Technology, MA, USA).

Blots were rinsed three times for 10min. before adding an appropriate, species specific
25 peroxidise labeled secondary antibody diluted in PBS. Following a further rinse step as described enhanced chemoluminescence was performed by adding 1ml of a commercial assay on to the blots for 5min. in the dark (#34075 West Dura Super Signal, Thermo scientific Fisher). Blots were developed in a dark room using autoradiography film (#28906836, HyperFilm™ GE Amersham, UK) and developer/fixer solutions. Blots were
30 stripped (#2502, Reblot Plus Mild Chemicon solution, Millipore) and reprobed for actin as described above.

The developed blots were dried and the ladder marked. The quantity of TRAIL in the bands was determined by normalising to actin and control samples using the
35 densitometry function on commercial software (Syngene SNAP software, Chemigenius2 bioimaging system, SynGene).

Analysis of miRNA Expression in Patients and controls

Whole Blood mRNA Extraction

The Sheffield Pulmonary Hypertension Biobank provided access to whole blood samples from patients (at time of diagnosis) and healthy volunteers under the local Sheffield Teaching Hospitals Foundation Trust Observational Cardiovascular Biobank Ethics 08/H1308/193 and STH15222). mRNA and miRNAs were extracted from Tempus (Applied Biosystems) whole blood samples according to manufacturer guidelines.

Quantitative real time Polymerase Chain Reaction - miR

Protocol

Quantitative PCR (qPCR) of mature microRNA (miR) was performed according to the manufacturer's instructions using TaqMan MircoRNA Assays. Briefly, total RNA was isolated from the lungs. cDNA was prepared using the TaqMan microRNA Reverse Transcription kit (4366596, Applied Biosystems) and qPCR performed using TaqMan microRNA Assays for U6 and miR-140-5p (Applied Biosystems). The primers used for reverse transcription and qPCR were those supplied with each assay. Each qPCR primer is a stem-loop oligonucleotide containing the sequence of the mature miR (miR-140-5p: CAGUGGUUUUACCCUAUGGUAG (SEQ ID No 1); U6: GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCA TGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT (SEQ ID NO 8)). qPCR was performed in triplicate using a 7900HT Fast Real-Time PCR System (Applied Biosystems). miR-140-5p levels were normalized to mean expression of the control group.

Reverse transcription of mRNA for first strand synthesis - protocol

This step was performed using components provided in a SuperScriptTM III first strand synthesis system (#18080-051 and #18080-044, InvitrogenTM Life technologies, UK). A volume containing 3µg of total RNA isolated from the lungs (and whole blood using PAX-gene tubes) of experimental rodents was made to 10µl using molecular grade water. 1µl of random hexamer primers (50ng) and 1µl of a 10mM dNTP were added to this and heated to 65°C for 5 minutes as a denature step. Samples were put on ice until 10µl of a cDNA synthesis mix [containing 10xRT buffer (2µl), 25mM MgCl₂ (4µl), 0.1M DTT (2µl), RNaseOUTTM (1µl) and SuperScriptTM III reverse transcriptase (1µl)] was added to this solution and mixed. Samples were heated in a thermal cycler (G Storm GS1, GRI Ltd, Essex, UK) with parameters set as follows i) 25°C for 10min. (annealing step), ii) 50°C for 50min. (cDNA synthesis) and finally iii) 85°C for 5min. before being held at 4°C- (to

terminate the reaction). 1µl of RNaseH was added to each tube before a final incubation step at 37°C for 20min.

Real Time quantitative PCR

- 5 Amplification of the target lung cDNA derived from the RT step above was then next performed. A volume containing 50ng of each cDNA was diluted to a volume of 4.5µl using sterile water. 5µl of a TaqMan® gene expression master mix-2X (#4369016, Applied Biosystems™ Life Technologies, UK) along with 0.5µl of the relevant target gene primers (10X) were added to the cDNA in the relevant well of a 384-well plate. Target
- 10 genes were tested are listed in table 1 (all from Applied Biosystems™) 18s and ATP5B were selected as endogenous control genes. Samples (in duplicate) for each gene were loaded on the same plate. The plate was centrifuged at 1000rpm for 1 min and the reaction was run on a 7900HT fast real time PCR system (Applied Biosystems™) using standard manufacturer settings. Relative expression for each gene was quantified by
- 15 comparing the test gene with the housekeeping control gene and comparing this ratio between an experimental and control subject (delta, delta CT method) for each gene using SDS software (v2.2.1, Applied Biosystems™).

Statistical analysis

- 20 Data were plotted and analysed using Prism® v6.0 (Graphpad, US) software. Data are expressed as Mean [standard error] unless indicated otherwise. Two groups were compared with Student's unpaired t test and three or more groups by ANOVA with Bonferroni post comparison testing (where indicated). Statistical significance was defined by a p value of ≤0.05.

25 Example 1

- Figure 1A shows miR-140-5p levels in whole blood samples taken at the time of diagnosis from patients with different forms of pulmonary hypertension and age and sex matched healthy volunteers. Levels of miR-140-5p are reduced in patients with multiple
- 30 forms of pulmonary hypertension. Figures 1B-D show that levels of miR-140-5p are further reduced in patients with more severe haemodynamic markers of disease compared to those with mild disease. Figure 1E shows that there is a significant survival effect of high verses low levels of miR-140-5p. Figure 1F shows a western blot of SMURF1, a putative target of miR-140-5p, 72 hours post transfection of human
- 35 pulmonary artery smooth muscle cells with 50 nM control miR (SCR), pre miR-140-5p

and anti-miR-140-5p. Figures 1G show that ID1, a downstream transcription factor from BMP signalling, and therefore influenced by SMURF1 is decreased in patients with IPAH. Figure 1H shows that SMURF1 is upregulated in patients with IPAH. Figures 1I-K show the time course of development of PAH in the monocrotaline rat. The rise in right ventricular systolic pressure (Figure 1I) and the development of right ventricular hypertrophy (Figure 1J) is preceded by a fall in miR-140-5p (Figure 1K). Figure 1L shows miR-140-5p expression from a time course in the Sugen+Hypoxia model of PAH and also demonstrates an early reduction in levels of miR-140-5p compared to normoxia controls.

10

Example 2

Figure 2A shows a reduction in the pulmonary artery acceleration time (PAAT) in monocrotaline treated rats with a scrambled (SCR) miRNA at 21 days compared to baseline. The PAAT was significantly increased in animals prophylactically treated with a pre-miRNA-140-5p agent. Figures 2B&C show the protective effects of pre-miR-140-5p prophylactic treatment in the monocrotaline rat model as demonstrated by reduced RVSP (B) increased cardiac output (C). Figure 2D demonstrates that the pre-miR-140-5p delivery did significantly rescue miR-140-5p levels in lung tissue, and this was associated with reduced levels of its target SMURF1 (E). Figure 2F shows a reduction in the pulmonary artery acceleration time (PAAT) in monocrotaline treated rats with a scrambled (SCR) miRNA at day 21 in the Pre-Rx group, and at day 35 in treated groups, compared to baseline. The PAAT was significantly increased in animals therapeutically treated with a pre-miRNA-140-5p agent. Figures 2G&H show the therapeutic effects of pre-miR-140-5p treatment in the monocrotaline rat model as demonstrated by reduced RVSP (G) increased cardiac output (H). Figure 2I demonstrates that the pre-miR-140-5p delivery did significantly rescue miR-140-5p levels in lung tissue. Figures 2G&H show the therapeutic effects of pre-miR-140-5p treatment in the Sugen+Hypoxia rat model as demonstrated by reduced RVSP (J) increased cardiac output (K). Figure 2L demonstrates that the pre-miR-140-5p delivery did significantly rescue miR-140-5p levels in lung tissue and this was associated with reduced levels of its target SMURF1 (M).

Example 3

Figure 3A shows proliferation of human pulmonary artery smooth muscle cells in 10% FBS with 10 ng/mL BMP4 at 72 hours post transfection with 30 nM control miR (SCR), pre miR-140-5p and anti miR-140-5p (n=5). Proliferation is significantly increased in the presence of anti miR-140-5p. Figure 3B shows proliferation of human pulmonary artery

smooth muscle cells in 0.2% FBS with 10 ng/mL BMP4 at 72 hours post transfection with 30 nM control miR (SCR), pre miR-140-5p, anti miR-140-5p and anti miR-140-5p with SMURF1 siRNA. Proliferation is increased with anti-mR-140-5p, an effect which is negated by the addition of SMURF1 siRNA.

5

Example 4

Figure 3C illustrates miR-140-5p targeting of PDGFRA expression. Cells transfected with the pre-miR-140-5p oligo display reduced levels of PDGFA therefore attenuating a PDGF response. Cells transfected with the anti-miR-140-5p, thereby mimicking the
10 disease state show increased expression of PDGFRA thereby increasing PDGF signalling.

Example 5

15 Figure 4 shows that treatment of established PAH with an anti-TRAIL antibody induces reverse remodelling of disease: ApoE^{-/-} mice after 8 weeks of feeding on the Paigen diet were implanted with osmotic pumps delivering either anti-TRAIL antibody of IgG for 4 weeks. Mice that received the anti-TRAIL antibody displayed a significant reduction in pulmonary vascular remodelling as shown by the reduction in media/CSA of the small
20 pulmonary arteries/arterioles (Figure 4A). This was associated with decrease in proliferating (PCNA) cells (Figure 4B) and an increase in apoptotic (TUNEL) cells within remodelled pulmonary arteries (Figure 4C).

Example 6

25 Figure 5 shows that treatment of established PAH with an anti-OPG antibody induces reverse remodelling of disease: ApoE^{-/-} mice after 8 weeks of feeding on the Paigen diet were implanted with osmotic pumps delivering either anti-OPG antibody of IgG for 4 weeks. Mice that received the anti-OPG antibody displayed a significant reduction in pulmonary vascular remodeling as shown by the reduction in media/CSA of the small
30 pulmonary arteries/arterioles (Figure 5A). This was associated with decrease in proliferating (PCNA) cells (Figure 5B) and an increase in apoptotic (TUNEL) cells within remodeled pulmonary arteries (Figure 5C). The Anti-OPG treatment demonstrates a more pronounced pro-apoptotic response than anti-TRAIL, which was associated with a greater anti-proliferative response.

35

Claims

1. A miR-140-5p, or polymorphic variant thereof, for use in the treatment of
5 pulmonary hypertension or conditions that result in pulmonary hypertension.
2. The miR according to claim 1 wherein miR-140-5p comprises the nucleotide
sequence CAGUGGUUUUACCCUAUGGUAG [SEQ ID NO: 1], or a polymorphic
sequence variant wherein SEQ ID NO: 1 is modified by addition, deletion or substitution
10 of at least one nucleotide base and which has retained or enhanced activity associated
with miR-140-5p.
3. The miR according to claim 1 or 2 wherein said miR-140-5p is a modified miRNA.
- 15 4. The miR miR-140-5p according to any one of claims 1 to 3 wherein the treatment
results in the regression of pulmonary hypertension.
5. The miR according to any one of claims 1 to 4 wherein pulmonary hypertension
is pulmonary arterial hypertension.
20
6. The miR according to claim 5 wherein pulmonary hypertension is idiopathic
(IPAH) or hereditary pulmonary hypertension (hPAH).
7. A pharmaceutical composition comprising miR-140-5p, or polymorphic variant,
25 and a pharmaceutically adjuvant and/or carrier.
8. The composition according to claim 7 wherein miR-140-5p comprises the
nucleotide sequence set forth in SEQ ID NO: 1, or a polymorphic sequence variant
wherein SEQ ID NO: 1 is modified by addition, deletion or substitution of at least one
30 nucleotide base and which has retained or enhanced activity of miR-140-5p.
9. The composition according to claim 7 or 8 wherein said pharmaceutical
composition includes a carrier adapted to deliver said miR to a subject in need of
treatment.

10. A combined pharmaceutical composition comprising miR-140-5p and at least one additional therapeutic agent used in the treatment of PAH.
11. The composition according to claim 10 miR-140-5p comprises a nucleotide
5 sequence set forth in SEQ ID NO: 1, or a polymorphic sequence variant wherein SEQ ID NO: 1 is modified by addition, deletion or substitution of at least one nucleotide base and which has retained or enhanced activity associated with miR-140-5p.
12. The composition according to claim 10 or 11 wherein said additional therapeutic
10 agent is selected from the group consisting of: calcium channel blockers, diuretics, endothelin receptor antagonists, prostacyclins, soluble guanalate cyclase and phosphodiesterase inhibitors.
13. The composition according to claim 10 or 11 wherein said additional therapeutic
15 agent is an antagonistic antibody, or active binding fragment thereof, that binds and inhibits the activity of Tumour Necrosis Factor Apoptosis-Inducing Ligand [TRAIL].
14. The composition according to claim 13 wherein said antibody competes with an
antibody that binds to the amino acid sequence as represented in SEQ ID NO: 2, 3 or 4.
20
15. The composition according to claim 14 wherein said antibody or binding fragment binds the extracellular domain of TRAIL.
16. The composition according to claim 15 wherein said antibody binds an epitope
25 located between amino acid residues 91–281 of the amino acid sequence presented in SEQ ID NO: 2.
17. The composition according to claim 10 or 11 wherein said additional therapeutic
agent is an antagonistic antibody, or active binding fragment thereof, that binds and
30 inhibits the activity of osteoprotogenin [OPG].
18. The composition according to claim 17 wherein said antibody competes with an
antibody that binds to the amino acid sequence as represented in SEQ ID NO: 5.
19. The composition according to any one of claims 13 to 18 wherein said antibody is
35 a polyclonal antibody.

20. The composition according to any one of claims 13 to 18 wherein said antibody is a monoclonal antibody.
- 5 21. The composition according to any one of claims 13 to 18 wherein said fragment is a single chain antibody fragment.
22. The composition according to any one of claims 13 to 18 wherein said antibody is a chimeric antibody.
- 10 23. The composition according to any one of claims 13 to 18 wherein said antibody is a humanized or human antibody.
24. A pharmaceutical composition according to any one of claims 7 to 23 which is adapted to be delivered as an aerosol.
- 15 25. An inhaler comprising a pharmaceutical composition according to claim 24.
26. A diagnostic method for determining if a subject has or is predisposed to pulmonary hypertension comprising:
- 20 i) providing an isolated biological sample to be tested;
- ii) forming a preparation comprising said nucleic acid to be detected in said sample, an oligonucleotide probe or probes adapted to anneal to a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 1; a thermostable DNA polymerase, deoxynucleotide triphosphates and co-factors;
- 25 iii) providing polymerase chain reaction conditions sufficient to amplify all or part of said nucleic acid molecule;
- iv) analysing the amplified products of said polymerase chain reaction for the presence of a nucleic acid molecule comprising a nucleotide sequence derived from SEQ ID NO: 1; and optionally
- 30 v) comparing the amplified product with a normal matched control.
27. A diagnostic method for determining if a subject has or is predisposed to pulmonary hypertension comprising:
- 35 i) providing an isolated biological sample to be tested;
- ii) forming a preparation comprising said nucleic acid to be detected in said sample, an oligonucleotide probe or probes adapted to anneal to a nucleic acid molecule

comprising a nucleotide sequence as set forth in SEQ ID NO: 6; a thermostable DNA polymerase, deoxynucleotide triphosphates and co-factors;

iii) providing polymerase chain reaction conditions sufficient to amplify all or part of said nucleic acid molecule;

5 iv) analysing the amplified products of said polymerase chain reaction for the presence of a nucleic acid molecule comprising a nucleotide sequence derived from SEQ ID NO: 6; and optionally

v) comparing the amplified product with a normal matched control.

10 28. The method according to claim 26 or 27 wherein said method is a real time PCR method for the detection and quantification of a nucleic acid encoding all or part of the nucleotide sequence set forth in SEQ ID NO: 1 or 6.

15 29. A diagnostic method for determining if a subject has or is predisposed to pulmonary hypertension comprising the steps of:

i) providing an isolated biological sample to be tested;

ii) forming a preparation comprising said sample and an antibody, or antibodies, that specifically bind a polypeptide in said sample as represented by the amino acid sequences presented in SEQ ID NO: 7 to form an antibody/polypeptide complex;

20

iii) detecting the complex; and

iv) comparing the expression of said polypeptide with a normal matched control.

25 30. The diagnostic method according to any one of claims 26 to 29 wherein said method further includes the detection of the expression of one or more genes associated with the BMP and/or PDGF pathway.

31. The diagnostic method according to claim 30 wherein said gene is selected from the group consisting of: SMURF1, WWP2, RUNX2, BMP, PDGFRA, OPG, SP1, TRAIL, ID1, TGRBR1 or VEGFA.

30

32. The method according to any one of claims 26 to 31 wherein said isolated biological sample is selected from the group consisting of: blood, blood plasma or serum, lymph fluid, saliva, sputum, lavage, bronchoalveolar lavage, or human tissue biopsy.

35

33. The method according to claim 32 wherein said biological sample is a blood sample.

34. The method according to claim 32 wherein said sample is a tissue biopsy.

5

35. The method according to any one of claims 26 to 34 wherein said method is combined with an alternative diagnostic method for the detection of pulmonary hypertension in a subject.

10 36. A method of treatment for PAH comprising:

i) conducting a diagnostic assay according to any one of claims 26 to 35 on a subject to be tested for PAH;

ii) determining whether the subject needs therapeutic intervention; and if requiring therapeutic intervention,

15 iii) administering a medicament or pharmaceutical composition according to any one of claims 7 to 23 to the subject.

37. The method according to claim 36 wherein said medicament or composition comprises miR-140-5p.

20

38. The method according to claim 36 or 37 wherein said subject is administered a second medicament or pharmaceutical composition comprising one or more agents used in the treatment of PAH.

25 39. The method according to claim 38 wherein said agent[s] are selected from the group calcium channel blockers, diuretics, endothelin receptor antagonists, prostacyclins, soluble guanalate cyclase and phosphodiesterase inhibitors.

30 40. The method according to claim 39 wherein said agent[s] is an antagonistic antibody, or active binding fragment thereof, that binds and inhibits the activity of Tumour Necrosis Factor Apoptosis-Inducing Ligand [TRAIL] or osteoproteogenin.

41. The method according to any one of claims 39 to 40 wherein said medicaments or compositions are administered simultaneously or sequentially.

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42. The method according to any one of claims 36 to 41 wherein pulmonary hypertension is pulmonary arterial hypertension.

43. The method according to claim 42 wherein pulmonary hypertension is idiopathic
5 (IPAH) or hereditary pulmonary hypertension (hPAH).

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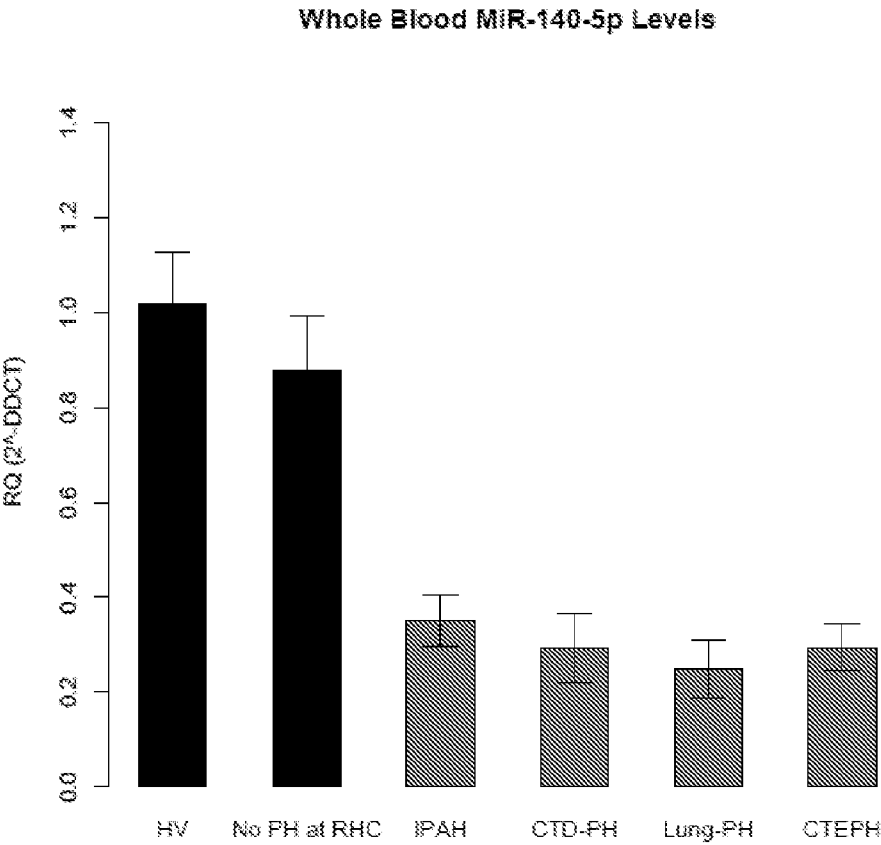
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30

35

Figure 1

A



B

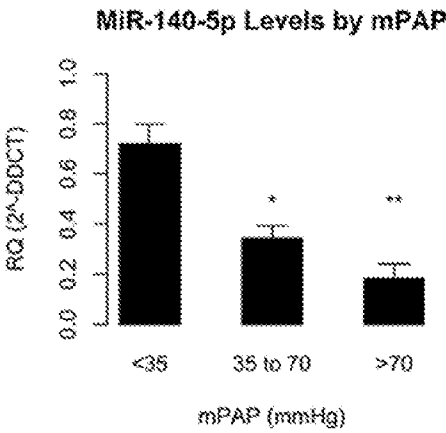


Figure 1 continued

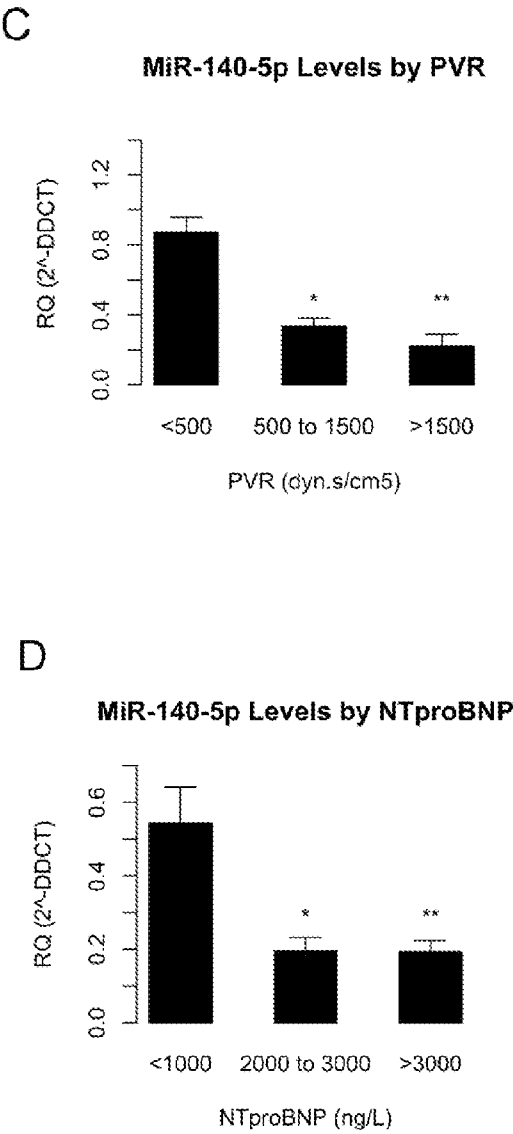
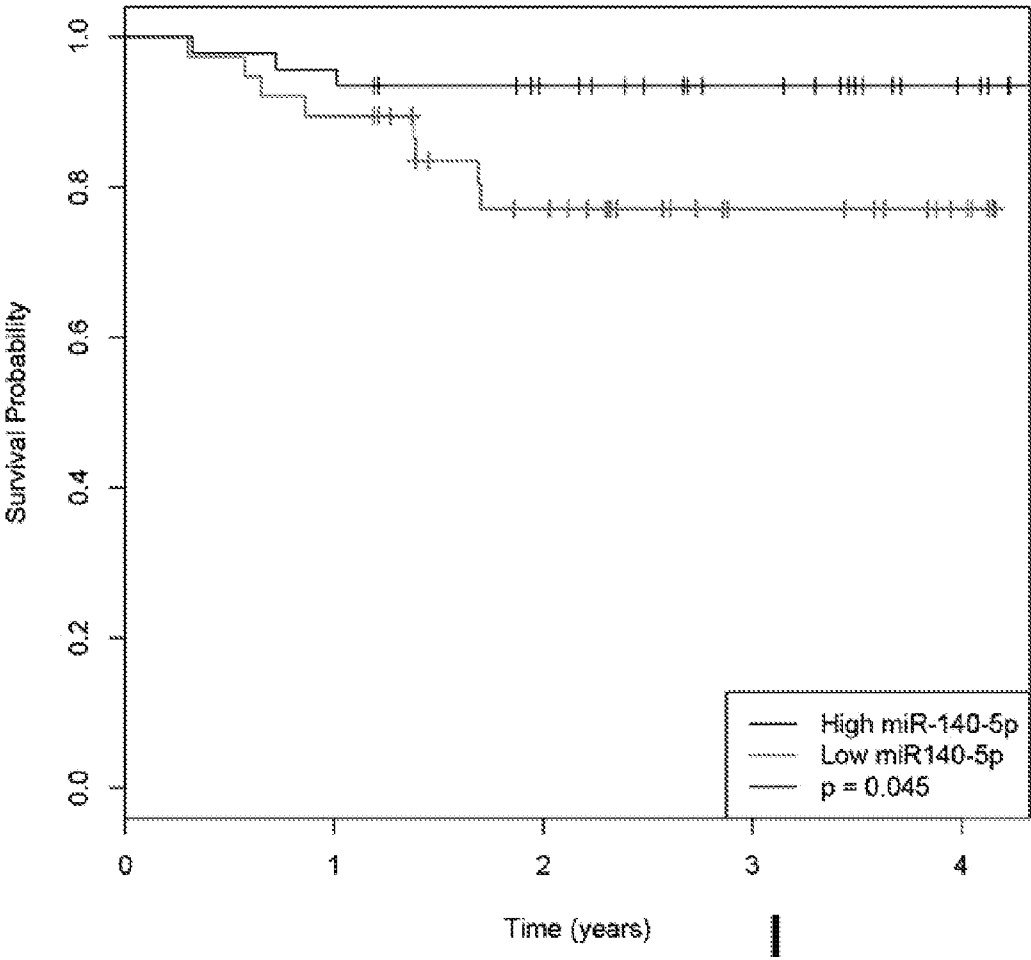


Figure 1 continued

E



F

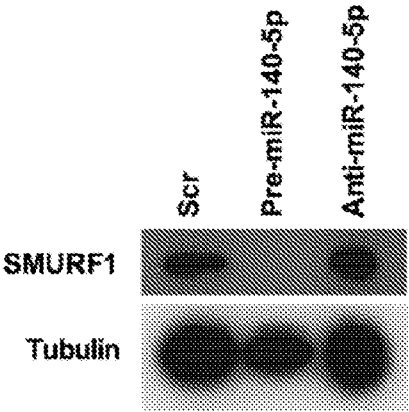
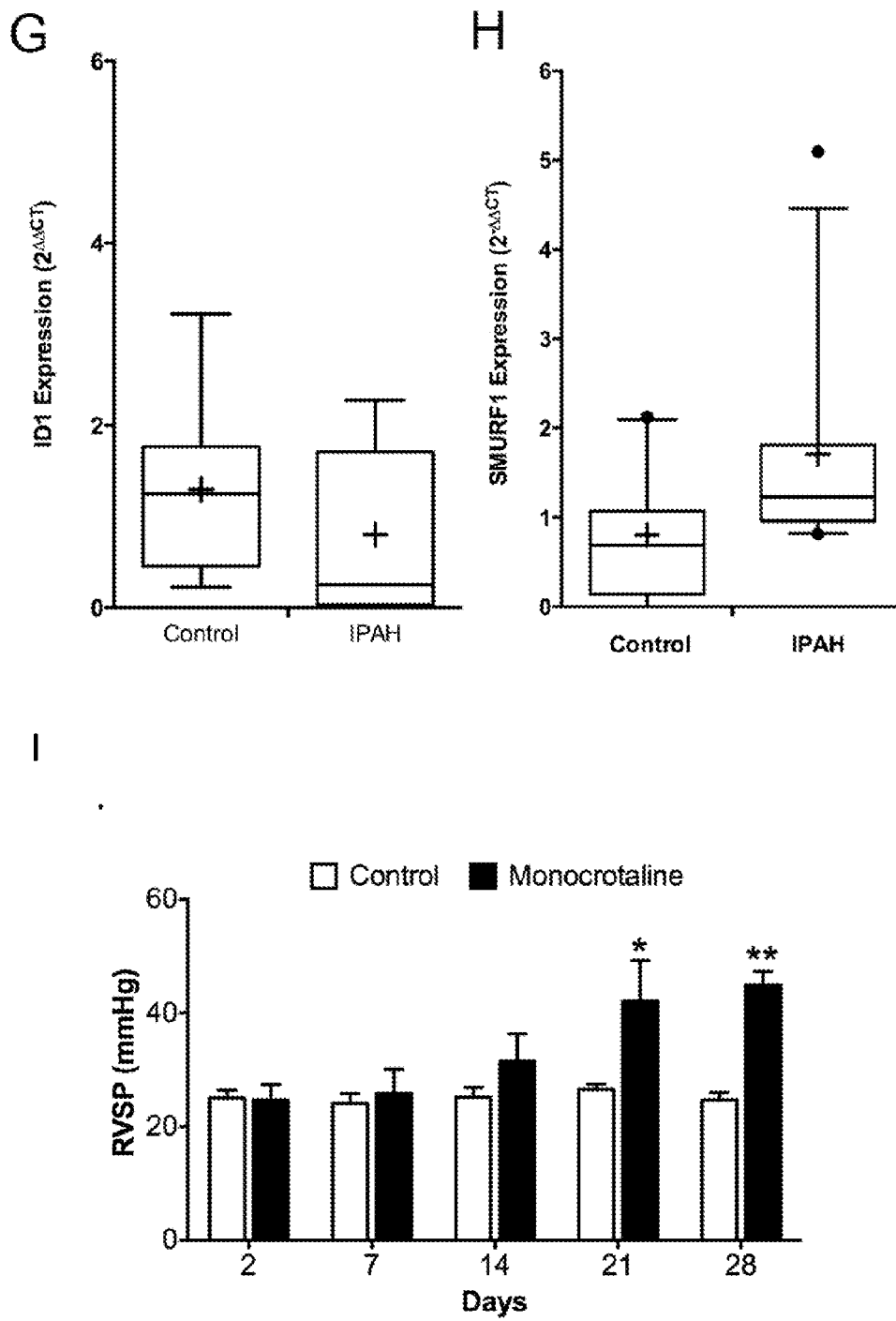


Figure 1 continued



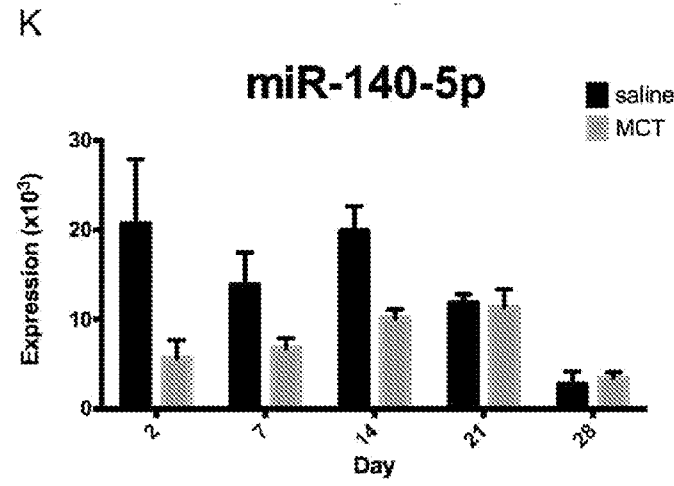
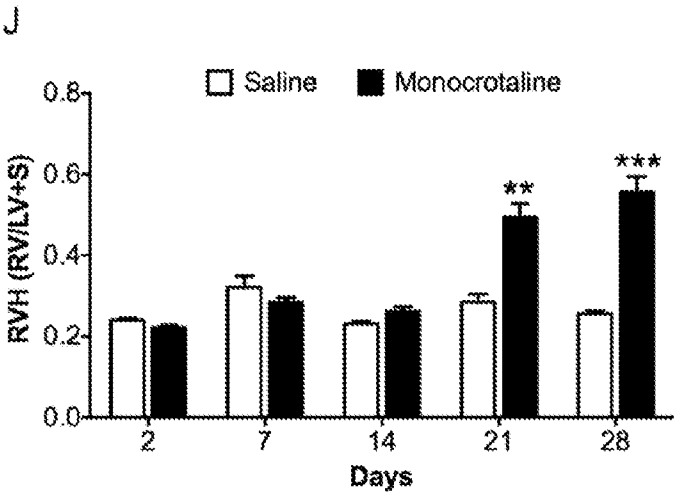


Figure 1 continued

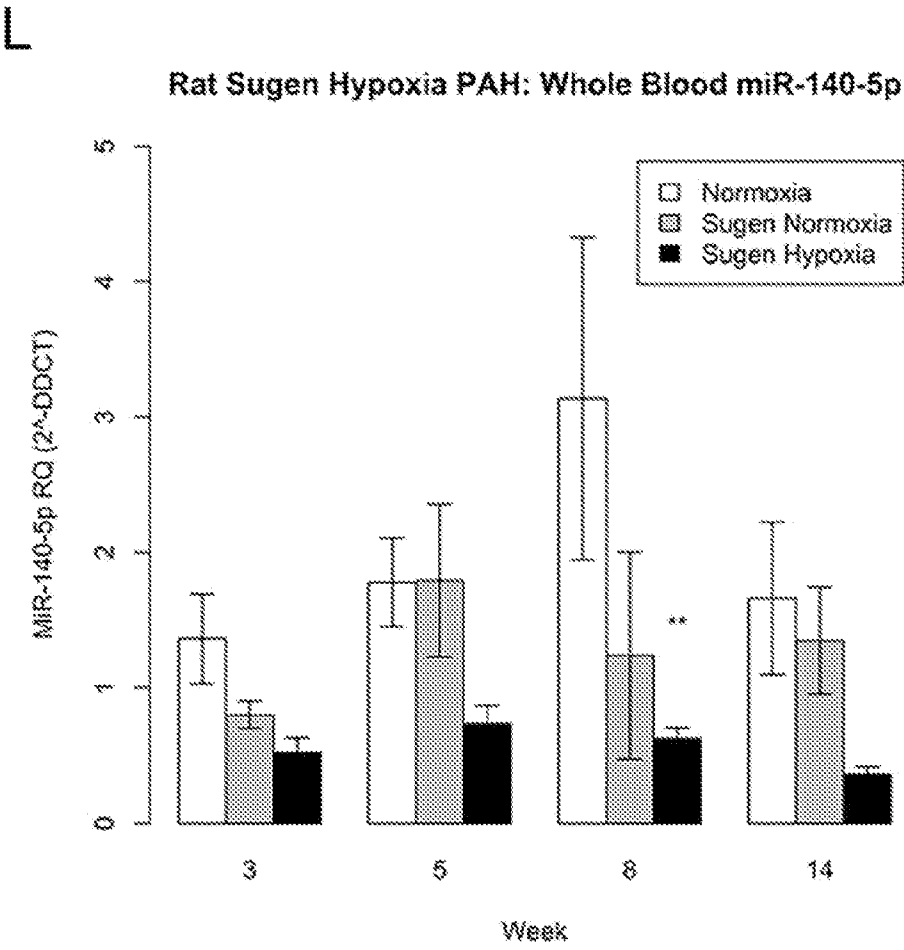


Figure 2

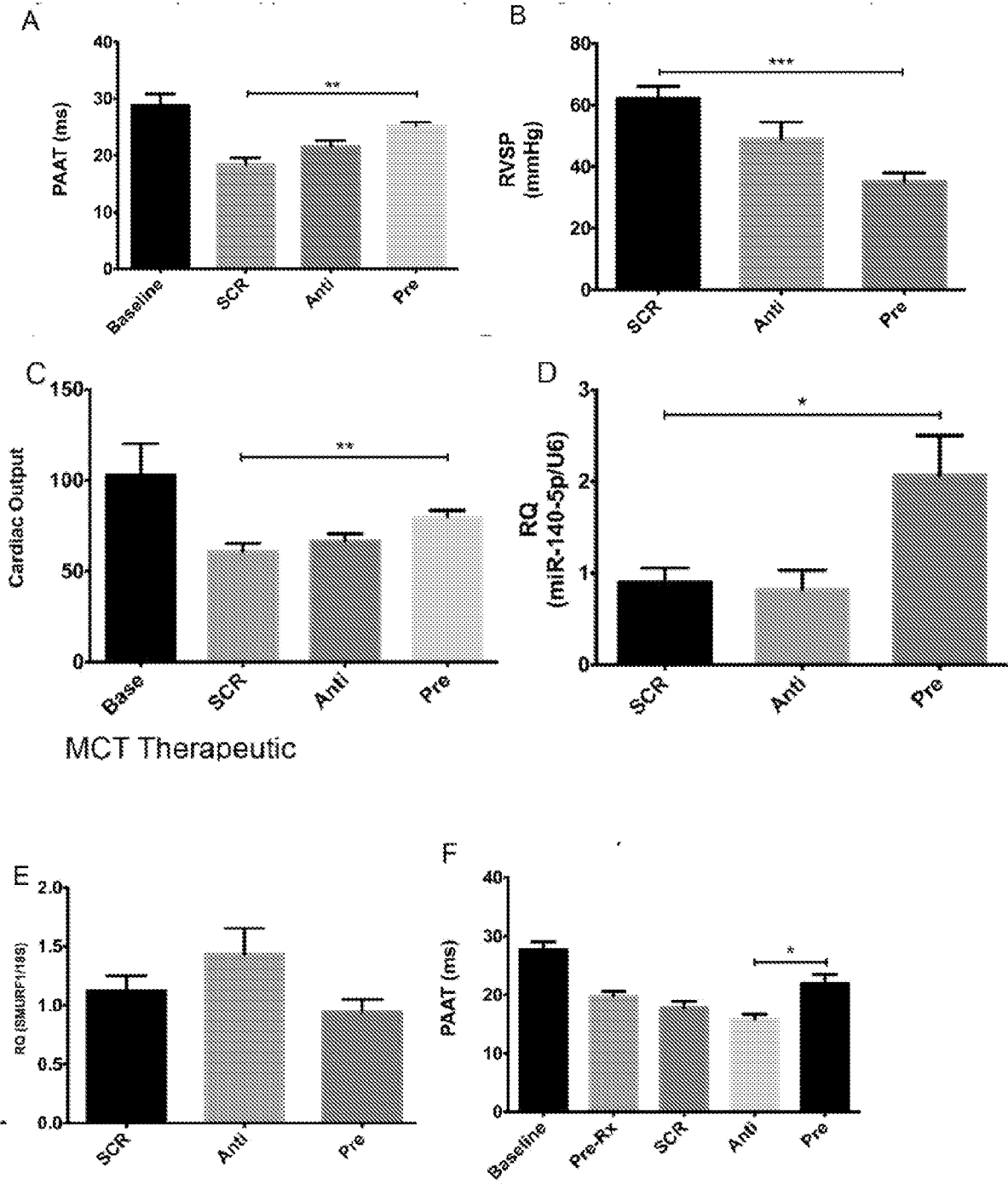


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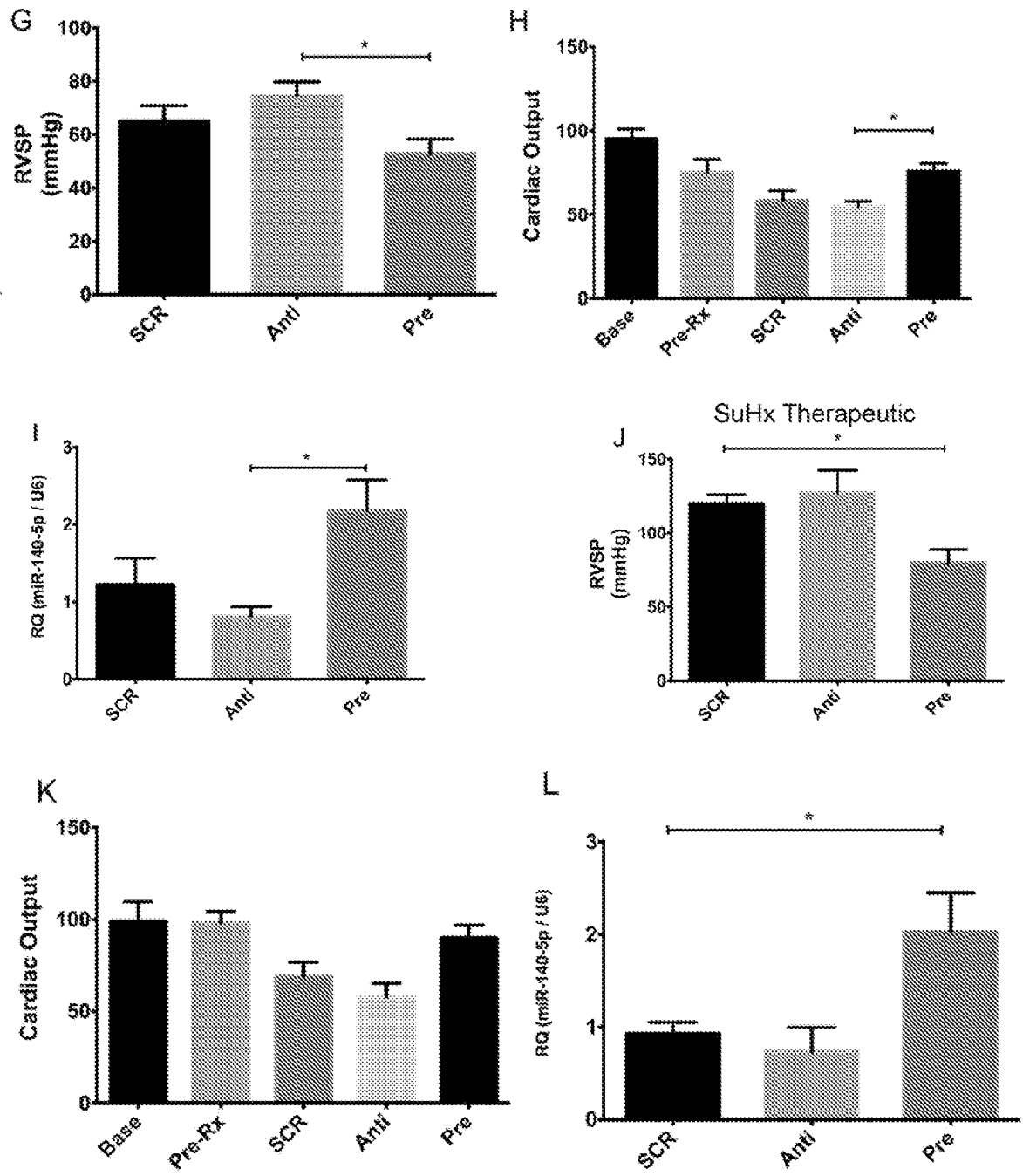


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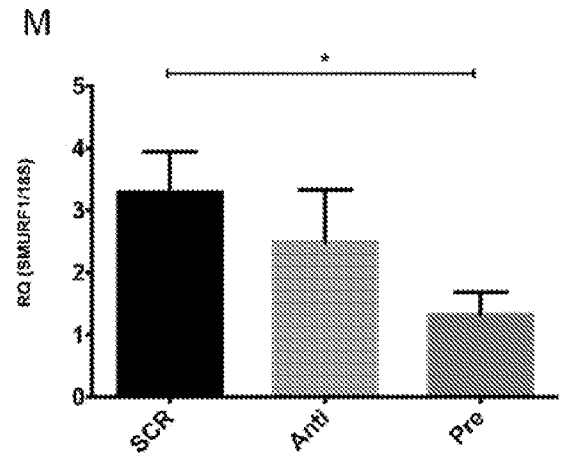


Figure 3

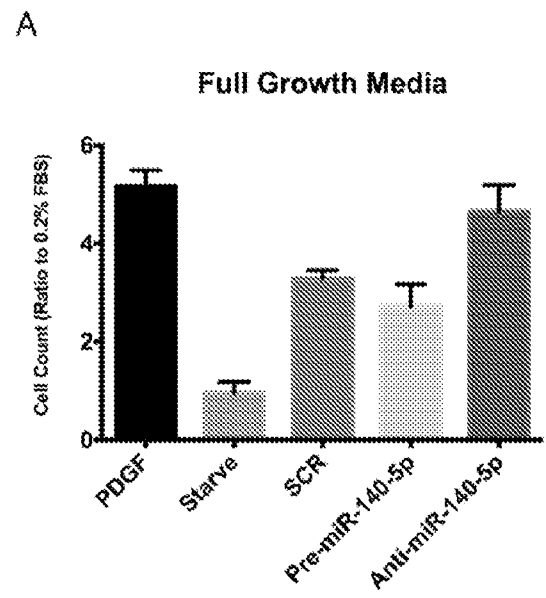
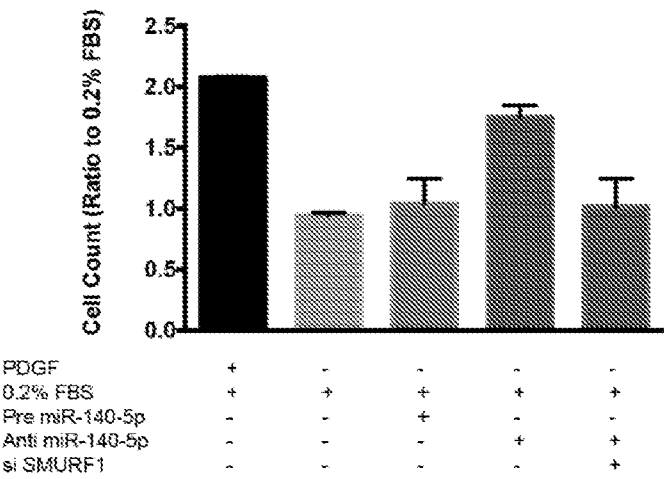


Figure 3 continued

B



C

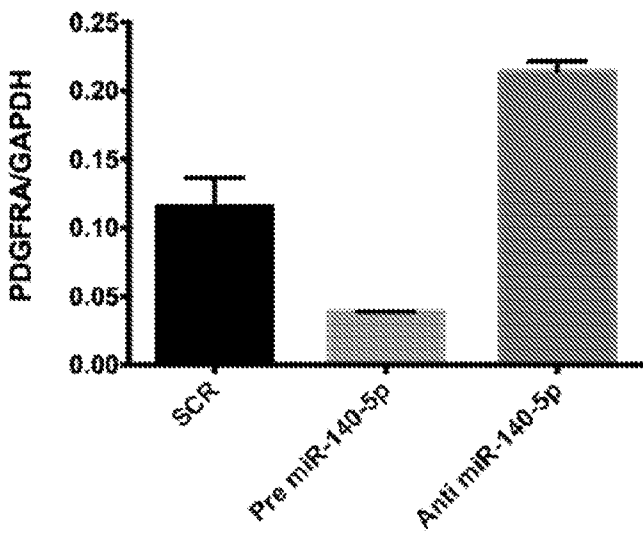


Figure 4

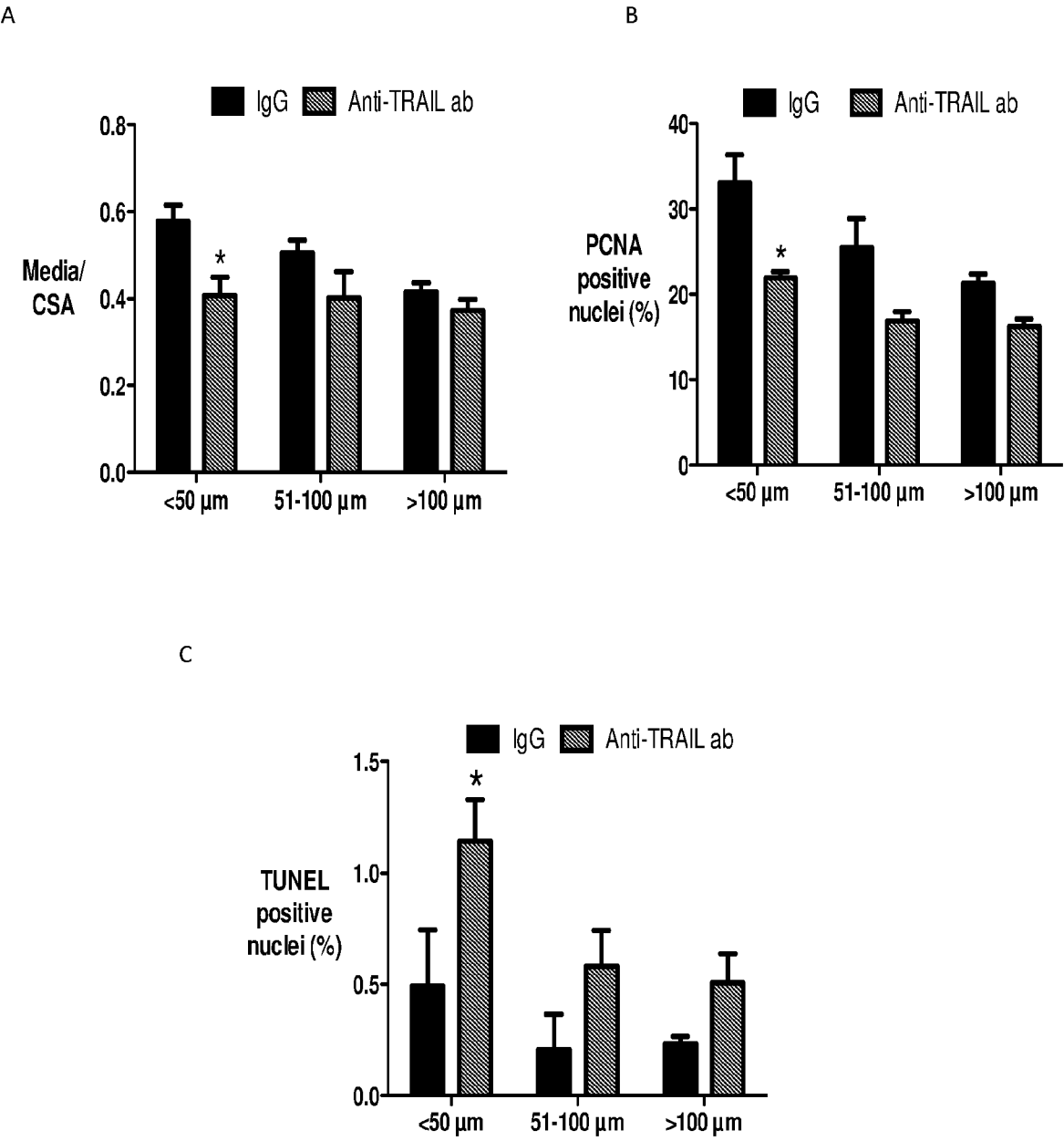
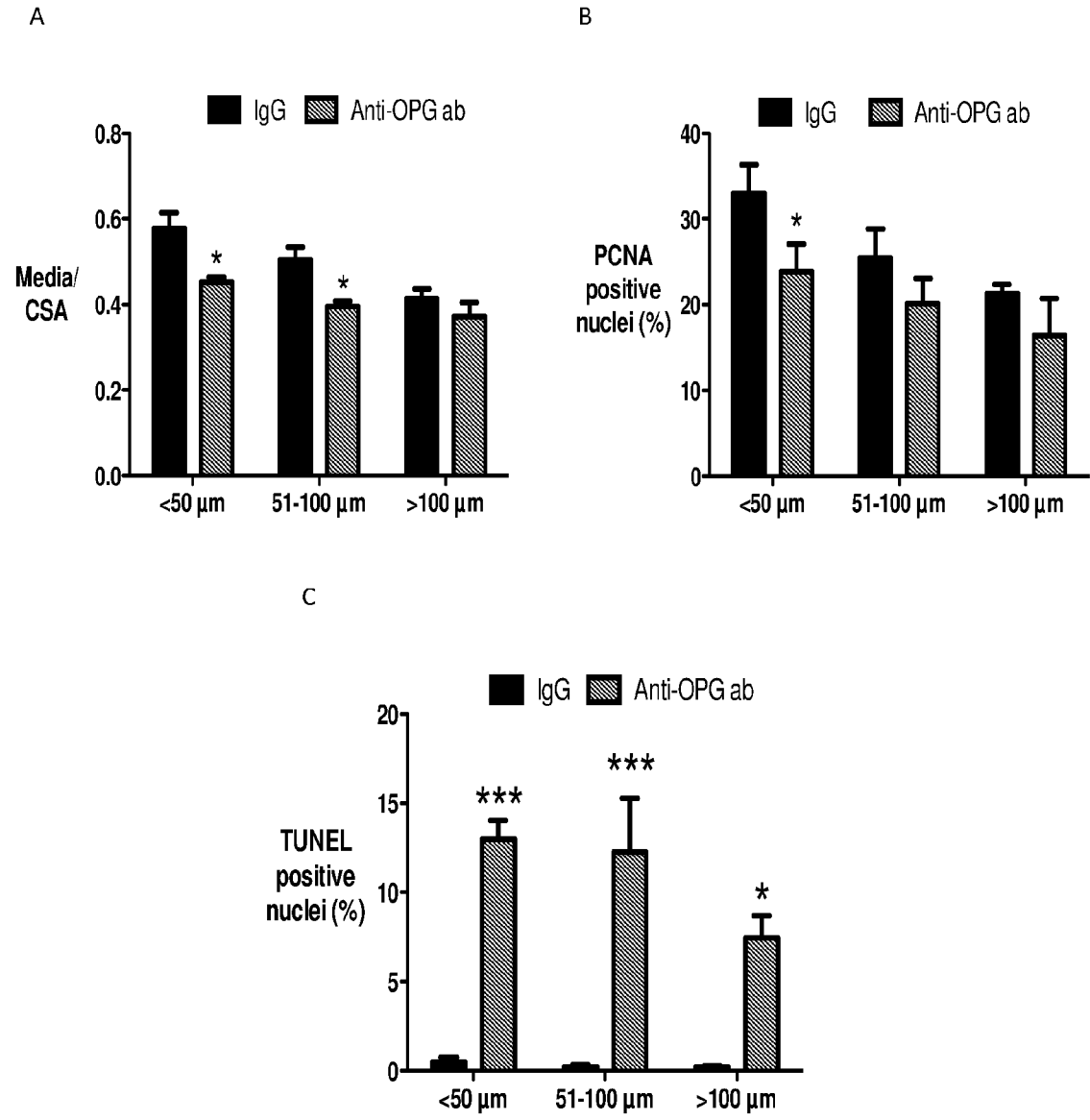


Figure 5



TRAIL isoform 1 (TNFSF10A):

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TRAIL isoform 2 (TNFSF10B):

SEQ ID NO: 3

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TRAIL isoform 3 (TNFSF10C):

SEQ ID NO: 4

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OPG

SEQ ID NO: 5

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NLPGTKVNAESVERIKRQHSSQEQT FQLLKLWKHQNKDQDIVKKIIQDIDLCENSVQRHIGHANLTFEQLRSLME
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PCR Primers

Gene	Human Primers	Rat Primers
U6	001973	001973
miR-140-5p	001187	001187
18s	HS03003631_g1	
SMURF1	Hs00410929_m1	
ID1	Hs03676575_s1	

SMURF1 cDNA Sequence NP_851994.1– 2196 nucleotides

SEQ ID NO: 6

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SMURF1 amino acid sequence: Uniprot Q9HCE7 - 731 amino acids

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/052683

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Alex Rothman: "Whole Blood MiRNA Expression in PAH Reveals Down-Regulation of MiRNA-140-5p", 30 January 2013 (2013-01-30), XP054975644, Retrieved from the Internet: URL:https://www.youtube.com/watch?v=6MVsoA -bt5E&feature=youtu.be [retrieved on 2014-12-12]	1-43
Y	the whole document ----- -/--	1-43



Further documents are listed in the continuation of Box C.



See patent family annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

19 January 2015

Date of mailing of the international search report

30/01/2015

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Bayer, Martin

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2014/052683

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Scientific Organizers ET AL: "Pulmonary Circulation Online Supplement: April-June 2013 Vol 3 No 2 Full conference report: The first Keystone Symposia Conference on pulmonary vascular disease and right ventricular dysfunction - Current concepts and future therapies", 1 September 2012 (2012-09-01), XP055158277, Retrieved from the Internet: URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3757848/pdf/PC-3-I.pdf [retrieved on 2014-12-12] Workshop 3; page 7	1-43
Y	----- WO 2013/061048 A1 (UNIV SHEFFIELD [GB]) 2 May 2013 (2013-05-02) cited in the application claims 1-29 examples 1-9	1-43
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A	----- CHUANYU WEI ET AL: "Circulating miRNAs as Potential Marker for Pulmonary Hypertension", PLOS ONE, vol. 8, no. 5, 23 May 2013 (2013-05-23), page e64396, XP055158310, DOI: 10.1371/journal.pone.0064396 the whole document -----	1-43

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2014/052683

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